

# THE ROLE OF THE INITIATOR CELL IN SLIME MOLD AGGREGATION<sup>1</sup>

MAURICE SUSSMAN<sup>2</sup> AND HERBERT L. ENNIS<sup>3</sup>

*Department of Biological Sciences, Northwestern University, Evanston, Illinois*

Previous studies of slime mold aggregation (Sussman and Noel, 1952) had shown that the number of aggregative centers is linearly related to the number of cells present and, further, that centers are distributed in accord with the Poisson series among small, replicate population samples. These and supporting data were considered to dictate the existence of specially endowed individuals termed "initiator cells," each of which could evoke the aggregative response by its neighbors, the "responder cells." Recently a distinctive cell type was detected by morphological criteria in *Dictyostelium discoideum* Raper and evidence was presented in support of the contention that cells of this type are in fact the initiators of aggregation (Ennis and Sussman, 1958a, 1958b; Sussman, 1958). The distinctive individuals, termed I-cells, are much larger than the remainder of the population (R-cells), the difference amounting to 2-3-fold in diameter, 3-10-fold in area. They are much flatter and more heavily granulated and vacuolated. In contrast to the R-cells which move sluggishly, the I-cells are highly motile and extensive lobopodia and filopodia are seen to protrude constantly and explosively. Figure 1 presents histograms to illustrate the size differences. Two modes are apparent without overlap.

The evidence (Ennis and Sussman, 1958b) supporting the candidacy of the I-cells for the appellation of "initiator" is summarized below:

- a) The ratio of I-cells to R-cells remained constant during the pre-aggregative period at 1:1940. This figure agrees closely with the ratio of centers formed to cells present at optimal density (1:2200).
- b) A high correlation was encountered between the positions of I-cells and of subsequently formed aggregative centers.
- c) The appearance of centers among small, replicate population samples was correlated (perfectly in one experimental series and almost perfectly in another) with the previously determined incidence of I-cells. That is, centers appeared in samples containing I-cells; none appeared in samples without I-cells.
- d) Removal of I-cells at an early enough time prevented subsequent center formation.

<sup>1</sup> This work was supported by grants from the National Cancer Institute and the Office of Naval Research.

<sup>2</sup> Present address: Department of Biology, Brandeis University, Waltham, Massachusetts.

<sup>3</sup> Postdoctoral Fellow, N.I.H. Present address: Department of Bacteriology and Immunology, Harvard University School of Medicine, Boston, Massachusetts.

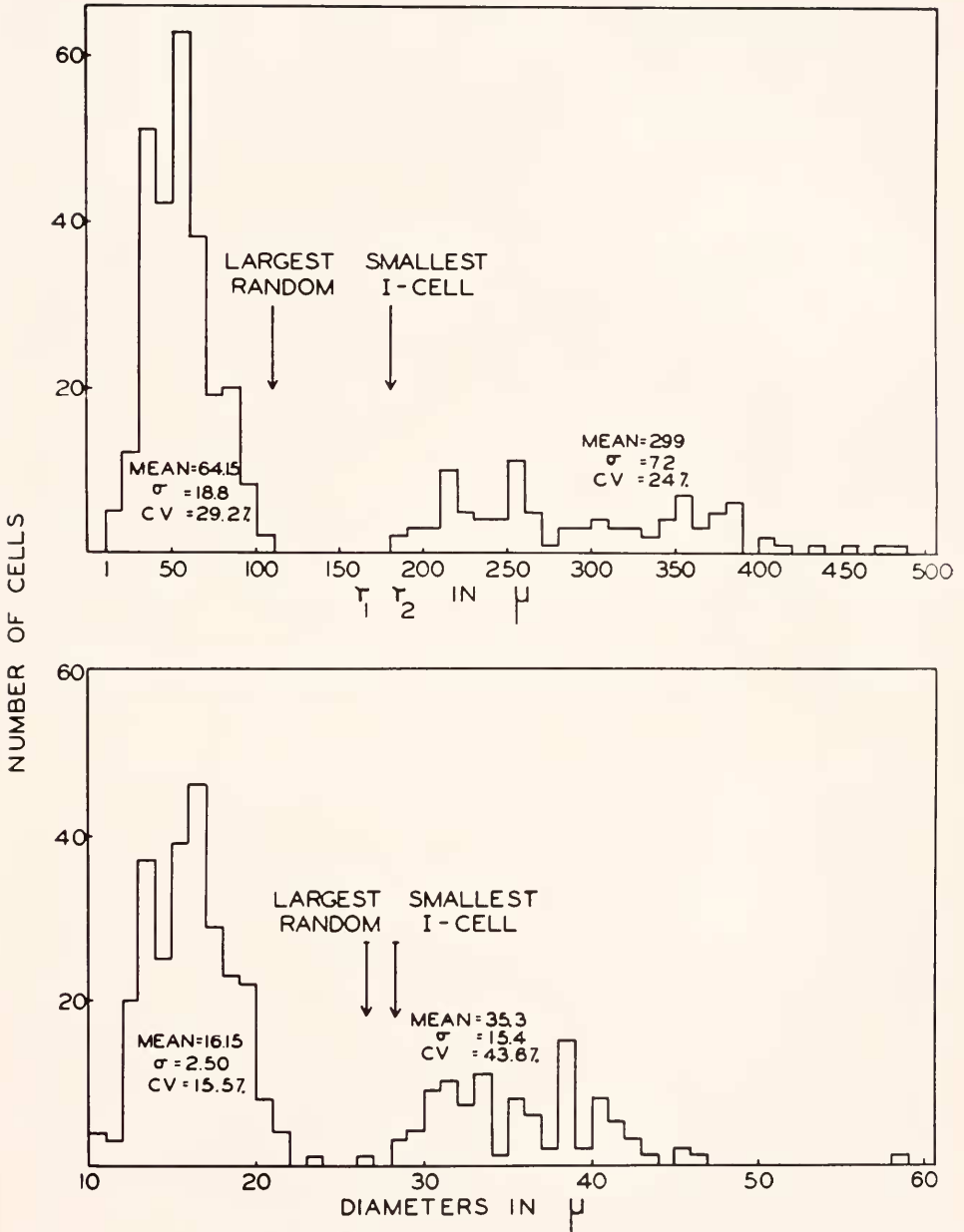


FIGURE 1. Histograms of mean diameters and products of major and minor radii. I-cells were detected under 100 $\times$  and confirmed under 440 $\times$  as described in the Methods section. As controls, myxamoebae were chosen at random for micrometric determinations.

- e) Micromanipulation of I-cells to test areas caused the induction of aggregates within the test populations, whereas movement of R-cells at the same stage of development did not.

The data to be presented provide subsidiary support for the contention that the I-cells are the initiator cells and throw light upon their role in the aggregative sequence.

#### METHODS

*D. discoideum*, strain NC-4 wild type, was grown on SM agar medium in association with *Aerobacter aerogenes* (Sussman and Noel, 1952). After 44 hours at 22° C., the myxamoebae had attained the stationary phase and were harvested, washed by differential centrifugation, and dispensed on washed agar plates (Sussman and Noel, 1952). Under these conditions the myxamoebae do not increase in number and aggregate and fruit in normal fashion.

The procedure for I-cell identification has been previously given in detail (Ennis and Sussman, 1958b). Initial recognition is accomplished at 100 × magnification by size and flatness. Examination at 440 × reveals the great pseudopodial activity, high rate of protoplasmic streaming, and granulation, and thereby confirms the diagnosis. A cautionary note is appended here. Occasionally, one encounters moribund cells which typically attain enormous size before lysing. However, these cells are perfectly round and hemispherical. They display no motility and have lost their granules and vacuoles. After one has seen an I-cell, there is no chance of confusing the two types and in any case, moribund cells are extremely rare under the conditions of preparation and incubation described above.

#### RESULTS

##### A. Time-lapse studies of aggregation

As mentioned, a high correlation was shown to exist between the positions of I-cells and of subsequently formed aggregative centers. In these experiments, washed myxamoebae were dispensed on washed agar at a population density of 200 cells/mm.<sup>2</sup>, optimal for center formation in this strain (Sussman and Noel, 1952). After 8 hours' incubation at 22° C., low power fields were chosen at random and fixed in position on microscope stages. Those found to contain I-cells were retained for further study. Camera lucida drawings or photomicrographs were made at intervals until aggregation had begun and the centers were established. In 50% of the fields, a center formed precisely at the position of the I-cell. (Since in this stock, the ratio of centers formed to cells present is 1:2200, the random chance of predicting that a center would form at a particular cell is 0.05%.) In 30% of the fields a center formed near the I-cell. No centers formed in the remaining 20%. In contrast, the incidence of centers in randomly chosen fields, not examined for the presence of I-cells, was 25%. Thus the over-all chance of a center appearing in a field containing an I-cell was three times greater than random. The numerical data, given in detail elsewhere (Ennis and Sussman, 1958b), are here amplified by time-lapse series of camera lucida drawings and photomicrographs.

Figure 2 illustrates the sequence of events when the center formed at the I-cell. The first overt sign of impending aggregation was the appearance of large cell clumps near the I-cell. Associations of more than two or three cells were never encountered prior to this time and even these were purely transient. In the series shown, the I-cell itself became part of a clump as its nearest neighbors began to



FIGURE 2. Time lapse camera lucida drawings of aggregation. The I-cell is the black individual. Cell clumps, appearing first in C, were merely outlined. The two cross-hatches mark the positions of dirt particles, used as points of reference. Respective times, in hours, after deposition on washed agar: 10.8, 11.25, 11.7, 11.9, 12.4, 13.0, 14.4.

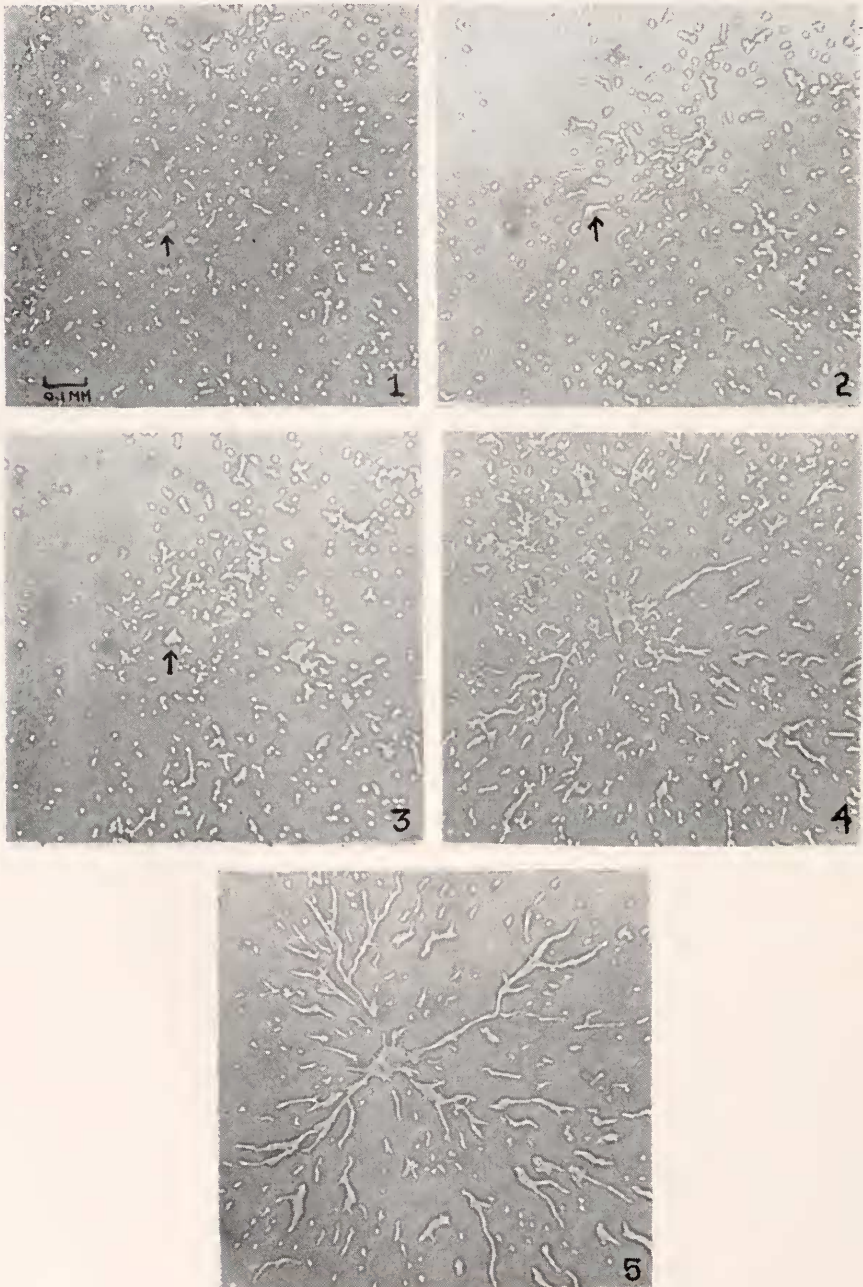


FIGURE 3. Time lapse photomicrographs of an aggregation. The arrows point to the I-cell. In photograph No. 2, the I-cell was joined by a few neighboring R-cells to form a tiny central clump. Photograph No. 3 was the last clearly discernible position of the I-cell.



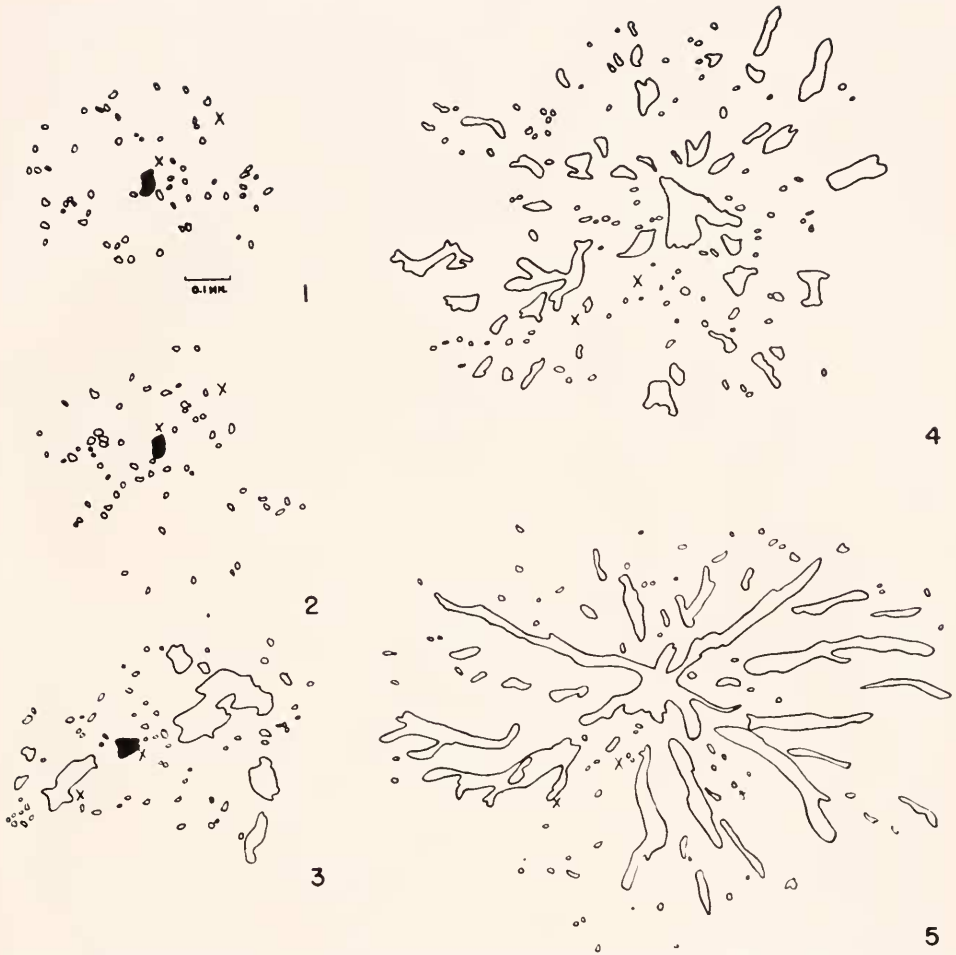


FIGURE 4. Time lapse camera lucida drawings of an aggregation. See legend of Figure 2 for details. Respective times, in hours after deposition on washed agar: 12, 12.5, 13, 13.4, 14.

nestle against it. This occurred in all but a few of the aggregations studied. The last clearly discerned position of the I-cell is in Figure 2-D. Its position was barely visible in Figure 2-E as the I-cell enlarged and extended to the right. Meanwhile, the previously formed clumps enlarged and new ones appeared concentrically about and at progressively greater distances from the I-cell. At this time, the loose cells and those in the clumps elongated and oriented radially. This caused the clumps to attain the appearance of streams. The position of the aggregative center then emerged clearly (Fig. 2-F) and is seen to have occupied the last known position of the I-cell. Ultimately the streams moved into and joined the center, producing the usual conical cell mass. Figure 3 is a series of photomicrographs of another aggregation in which the center again formed at the I-cell.

A typical sequence in which a center was established near the I-cell is shown in Figure 4. Again, the first sign of impending aggregation was the appearance of cell clumps, although on this occasion, no clump formed around the I-cell. A particularly large clump appeared at a distance of about 200  $\mu$  from the I-cell (Fig. 4-C). The I-cell then moved into a small clump immediately above the upper right reference mark. The cells elongated and oriented radially and a center was established at a distance of about 100  $\mu$  from the last known position of the I-cell. Thus the only real difference between the sequences shown in Figure 2 and Figure 4 is the appearance of the abnormally large clump, and this event always preceded the establishment of a center near, rather than at, the I-cell. In 10 of 19 cases, the R-cells entered the aggregate but the I-cell remained outside. In the other 9 cases the I-cell was also swept into the aggregate.

Figure 5 shows that a center need not form at a point along the previous path of the I-cell. The four cases were chosen because the respective I-cells wandered along relatively straight paths and could therefore clearly illustrate that such a relation did not exist. Cases 3 and 4 are particularly pertinent in that the centers did not form at the I-cells but did so at distances of 250 and 300  $\mu$ , respectively.

### B. Aggregation after I-cell removal

The fact that I-cell removal can prevent subsequent aggregation was established in previously reported experiments (Ennis and Sussman, 1958b). They also showed that, to be effective, the removal must be accomplished at a very early stage of the pre-aggregative period. Thus, I-cells were removed from drops containing 500 myxamoebae within two minutes after they had been dispensed on washed agar. The incidence of centers 16 hours later (at which time all aggregations were completed), was only 9% of the incidence in the control drops from which I-cells had not been removed. If, however, the I-cells were permitted to remain for about 5 minutes before removal, the incidence of centers rose to 40% of the control value. Removal at 20 minutes increased the incidence to 67% of the controls and removal at one hour was totally ineffective, *i.e.*, equal percentages of aggregates developed in the controls and in populations from which I-cells were removed. Clearly, then, the presence of the I-cell in the immediate vicinity of the R-cells, even for a few minutes, is sufficient to produce an inductive effect.

Experiments performed since then have indicated that the I-cells can exert this inductive effect upon R-cells well outside of their immediate vicinity, albeit they require more time to do so. Replicate samples of 6000 washed myxamoebae were dispensed on washed agar at the optimal density of 200 cells/mm.<sup>2</sup>. The excess fluid was absorbed by the agar and after one hour's incubation, an area, 1 mm.<sup>2</sup>, was delineated at the center of each drop by scoring the agar surface lightly with two pieces of razor blade, mounted parallel at a distance of 1 mm. The cells outside of the square were brushed away, thereby leaving replicate samples of 200 myxamoebae at a density of 200 cells/mm.<sup>2</sup>.

Since the distribution of I-cells has been found to be 1:1940, one would expect about 10% of the squares to have contained I-cells and accordingly to have aggregated. As may be seen in Table I, precisely 10% of the squares so treated did aggregate. Thus, it can be said that all of the aggregates observed must have been contributed by those squares that contained I-cells and that no I-cells lying

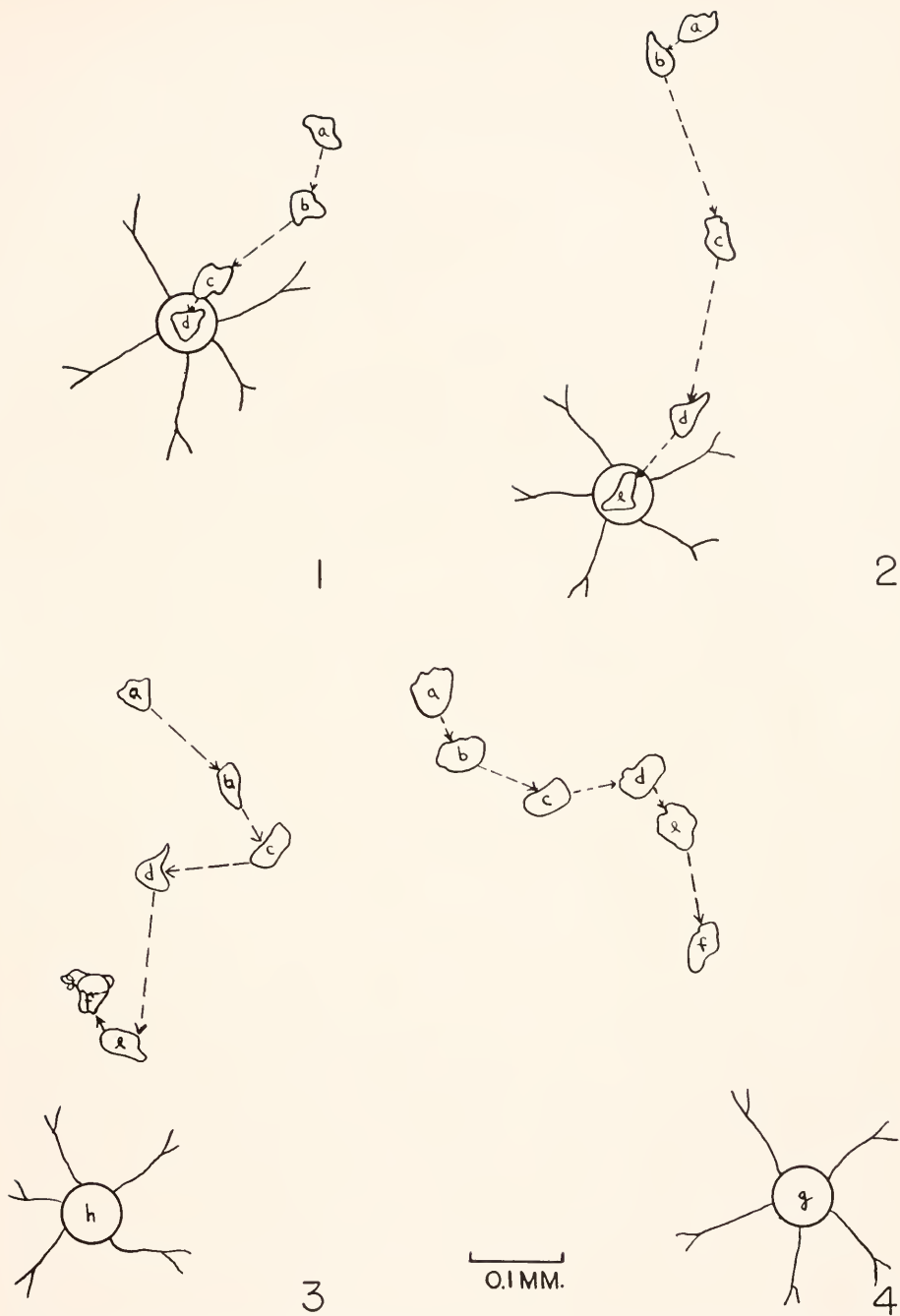


FIGURE 5. Relation between aggregative centers and previous migratory pathways of I-cells in four aggregations. In the top two, the centers coincided with the final positions of the I-cells. In the bottom two, they did not. The respective times, in hours, at which the first and last camera lucida drawings were made after deposition on washed agar: 10.8, 11.9; 9.8, 12.25; 9.8, 14.0; 10.0, 13.0.



TABLE I

*After the stated periods of pre-incubation, squares were scored on the agar surface and outlying cells were brushed away. See text for details.*

Pre-incubation period in hours	No. of squares	No. with aggregates	%
1	120	12	10.0
4-5	120	20	16.7
6-8	120	59	49.0
10-12	120	109	91.0

outside the boundaries of said squares for the one-hour period and then brushed away, appear to have exerted an inductive effect upon the R-cells lying within. When, however, a period of 4-5 hours elapsed before the squares were delineated and the outlying cells removed, the incidence of aggregates within the square rose to 16.7%. After 6-8 hours the incidence was 49% and after 10-12 hours, 91%. (At 12 hours, but not at 10, the cells were elongated and were beginning aggregation.) Thus, even at 4-5 hours, significantly more aggregates appeared than could be accounted for simply by the presence of I-cells within the squares. The data would therefore seem to force the conclusion that the I-cells lying outside the squares must have exerted an inductive effect upon the R-cells within. It is important to note that, since the total cell density was 200 cells/mm.<sup>2</sup>, the density of the I-cells would have been about 1 per 10 mm.<sup>2</sup>. Thus the I-cells in exerting their effect acted over truly fantastic distances.

The objection may be raised that not only the outlying I-cells were removed but also the outlying R-cells. Why, then, could one ascribe the inductive effect to the latter? The answer lies in the fact that when replicate samples of from 250 to 2000 myxamoebae were dispensed at densities even greater than 200 cells/mm.<sup>2</sup>, a Poisson distribution of aggregates was obtained in strict accordance with the distribution of I-cells. If R-cells had any inductive capacity of their own at these densities, why then did not every sample aggregate regardless of whether an I-cell was present or not?

In summary, the data indicate that the I-cell performs its mission at the early stages of the pre-aggregative period. The immediate neighbors of the I-cell require its presence for only a few minutes and can then subsequently aggregate in its absence. The more remote neighbors can also be affected if the I-cell is allowed to remain for a longer period of time. It is difficult to explain these results save by the assumption of a diffusible "initiator substance."

### C. *The initiative capacity of R-cells*

As mentioned previously, when I-cells were micromanipulated to test areas, they could induce the test populations to aggregate, whereas R-cells at the same stage of development could not (Ennis and Sussman, 1958b). In these experiments, the I-cells and R-cells were micromanipulated to the test areas within 20 minutes after they had been dispensed on washed agar. The question arose as to whether or not R-cells which had been incubated for periods longer than 20 minutes prior to micromanipulation might not display initiative capacity.

TABLE II

After the stated periods of pre-incubation, R-cells were individually micromanipulated to test areas. See text for details

Pre-incubation period in hours	Experimental			Background		
	Total	No. with aggregates	%	Total	No. with aggregates	%
1	53	7	13.2	79	11	13.9
4-6	65	14	21.6	250	29	11.6
10-12	71	26	36.6	70	9	12.8

Washed myxamoebae were dispensed on washed agar at a density of 150-200 cells/mm.<sup>2</sup>. After 1, 4-6, and 10-12 hours, R-cells were picked up individually with a glass loop mounted in a deFonbrune micromanipulator and moved to test areas. The test areas had been prepared by dispensing washed myxamoebae on washed agar at a density of 250 cells/mm.<sup>2</sup>, one hour prior to use. After the excess fluid had been absorbed, an area, 1 mm.<sup>2</sup>, was delineated in the middle of each drop as described in the previous section. The outlying cells were brushed away leaving test squares containing 250 myxamoebae at a density of 250. The center:cell ratio being 1:2200, one would expect 11.3% of the squares to have aggregated spontaneously. The background controls shown in Tables II and III showed an incidence of 72 squares with aggregates out of a total of 578, or 12.4%. The extent to which addition of R-cells, pre-incubated for periods between 1 and 12 hours, affected the background incidence is shown in Table II. R-cells pre-

TABLE III

Initiative capacity of R-cells tested upon their developmental juniors

A. Samples with I-cells			Samples without I-cells		
No.	No. with aggregates	%	No.	No. with aggregates	%
21	18	86	13	0	0

B. Experiment	R-cells from samples with I-cells			R-cells from samples without I-cells			Background		
	Total	No. with aggregates	%	Total	No. with aggregates	%	Total	No. with aggregates	%
A	27	5	18.5	27	8	29.6	54	8	14.8
B	27	8	29.6	27	3	11.1	53	8	15.1
C	36	8	22.2	30	7	23.3	72	7	9.7
Total	90	21	23.4	84	18	21.4	179	23	12.8

A. Samples of 500 cells were dispensed on washed agar. Twenty-one which certainly contained I-cells and 13 which certainly did not were chosen. The percentages of samples that produced aggregates are shown.

B. After 8 hours' pre-incubation, R-cells, taken from the samples with and without I-cells, were micromanipulated to test areas. See text for details.

incubated for one hour did not affect the background frequency but increases of 10 and 24% over background were obtained by adding R-cells pre-incubated for 4-6 and 10-12 hours, respectively. In other words, when pre-incubated for 10-12 hours and then moved to test areas, one out of four R-cells could induce the formation of a center among the test cells, 12 hours after its introduction.

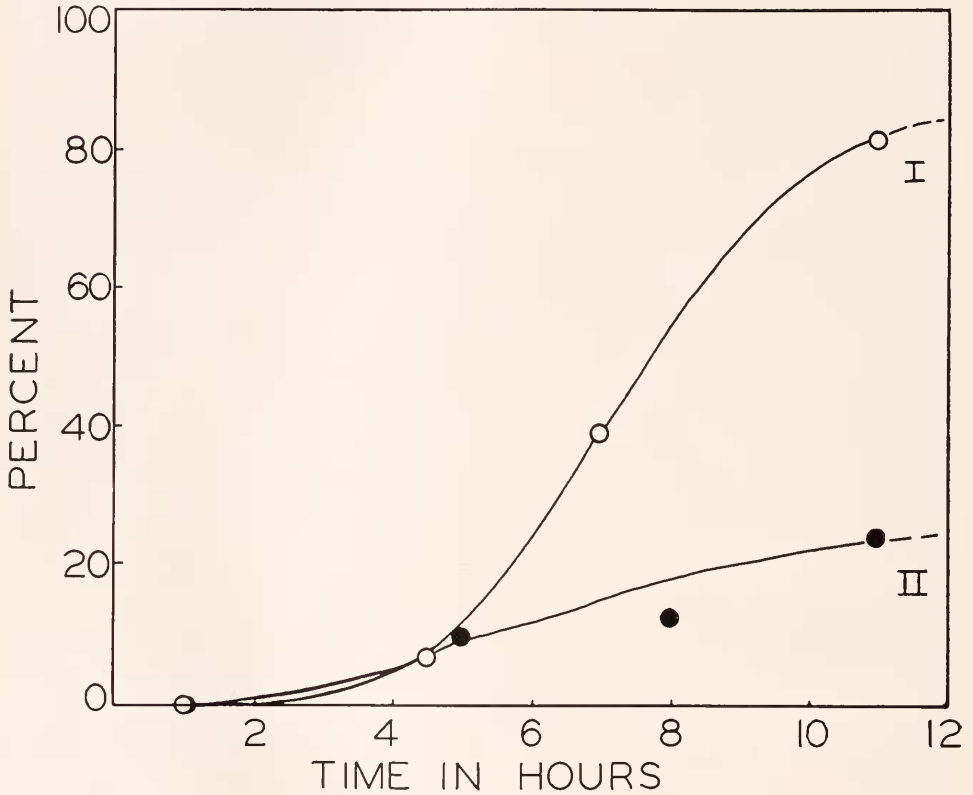


FIGURE 6. A kinetic comparison of: I. The capacity of small population samples to aggregate when isolated from their neighbors after varying periods of incubation. Ordinate: per cent of 250 cell samples that aggregated. Abscissa: time of incubation on washed agar prior to isolation. (Data from Table I.) II. The capacity of R-cells incubated for varying times on washed agar to initiate centers amongst their developmental juniors. Ordinate: per cent of R-cells capable of initiation. Abscissa time of incubation on washed agar prior to their micromanipulation to test areas. (Data from Table II.)

Figure 6 is a graphic comparison of the kinetics of induction of centers in test squares (I) by progressively delayed removal of outlying I-cells (data from Table I) and (II) by addition of pre-incubated R-cells (data from Table II). The crude kinetic similarity suggested that the outlying I-cell might not only be responsible for the subsequent aggregation of the R-cells but also for the concomitant increase in their capacity to themselves initiate centers. To test this possibility, replicate samples

of 500 washed myxamoebae were dispensed on washed agar. In three experiments 21 samples were chosen which certainly contained I-cells and 13 which certainly did not. The data in Table III confirm the correctness of these choices since 86% of the samples said to contain I-cells aggregated while none of those said not to contain I-cells did so. After these samples had been incubated for 8 hours, R-cells were picked and moved to test squares as described in the preceding paragraph. Table III shows that R-cells, whether pre-incubated in the presence or absence of I-cells, were equally capable of inducing center formation. Thus, the rise of the initiative capacity of the R-cells during the pre-aggregative period is not dependent upon their contiguity with I-cells. Two points must be kept in mind here. First, it must be remembered that prior to their deposition on the washed agar, R-cells had all been in contact with I-cells and therefore could have been at this time the subject of interactions emanating from the latter. Second, even though the R-cells after 12 hours of incubation had attained a significant degree of initiative capacity, they fell far short of the level displayed by the I-cells after only 20 minutes of incubation. Therefore, the phenotypic difference between the two cell types in this respect remains clear.

Finally, the results reveal a most puzzling paradox. When R-cells were pre-incubated for 8 hours in the absence of an I-cell and then placed in the presence of test cells for an additional 12 hours, at least one out of ten could induce center formation. Yet the samples from which these R-cells originally came, when incubated for a total of 20 or indeed 36 hours, had not aggregated. It is clear, therefore, that the observed increase in the initiative capacity of R-cells during the pre-aggregative period in the development of a population is of no consequence to the ultimate aggregation of *that* population. In other words, the initiative capacity of such R-cells, demonstrated by movement to another population, is an experimental artifact bearing no relation to normal aggregation but which may possibly be used to understand the biochemical and genetic differences between the I-cell and R-cell *Phenotypes*.

## DISCUSSION

The data presented here and previously suggest a developmental program of slime mold aggregation that may serve as a useful working hypothesis.

I-cells arise during the growth of an R-cell population (which in turn had originated from the spores of the preceding fruit), and attain a steady-state ratio of approximately 1:2000 early in the exponential phase (Sussman, 1956; unpublished data). Entrance into the stationary phase marks the beginning of the pre-aggregative period. At the beginning of this period, the I-cells secrete material which, during the ensuing 12 hours, so conditions the neighboring R-cells as to induce them to aggregate. This interaction, as might be expected, affects the nearest neighbors first but its influence is progressively extended. Concomitant with, but unrelated to either the presence of the I-cell or the subsequent course of aggregation in the same population is a significant rise in the initiative capacity of the R-cells themselves. Such cells upon extended incubation never do attain the degree of initiative capacity displayed by the I-cells nor can they act upon their developmental contemporaries but only upon cells at an earlier developmental stage to which they have been added by the observer.

The first overt sign of aggregation is the formation of cell clumps concentrically about and usually at the I-cell. This is followed by excitation and elongation of the loose and clumped cells in response to the chemotactic complex (Sussman *et al.*, 1956; Shaffer, 1956; Sussman, 1958). The appearance of oriented streams establishes the position of the aggregative center. This is usually coincident with the final position of the I-cell but sometimes with the position of a particularly large clump nearby, and possibly reflects the point of greatest production of the chemotactic complex. In the latter case, the position of the center need bear no relation to the previous path of the I-cell.

The picture as drawn raises many questions and offers a number of predictions under current study. The most important of the latter involves the hypothetical existence of an "initiator" substance. In view of the I-cell removal experiments, one ought under the same conditions to be able to induce test cells to aggregate by dispensing them in an area previously but no longer occupied by an I-cell. This is being tested. The I-cell addition experiments raise the question as to what is the minimum period of time after contact with the I-cell in which the induced R-cells can begin aggregation. Is the 12-hour period subsequent to contact mandatory or does it involve preparations by the R-cells for aggregation, unconnected with the function of the I-cell? In the latter case, one ought to be able to pre-incubate the test cells for twelve hours, add I-cells, and observe the onset of aggregation very shortly thereafter.

The fact that R-cells can also attain initiative capacity to a far smaller degree, albeit much later than do the I-cells and ineffectively so far as inducing their contemporaries to aggregate is concerned, still suggests that the metabolic pathways involved in initiation are not unique to the I-cells. Indeed, one may imagine that the sole basis for the difference between I-cells and R-cells in this respect is the much greater size of the former. Perhaps, then, any of the diverse methods for producing giant cells may serve to create initiators just as does the normally occurring R-cell to I-cell transformation. This point is also under current study.

#### SUMMARY

*Dictyostelium discoideum* myxamoebae occur as two distinct morphological types, termed I-cells and R-cells. Data presented in a previous publication demonstrate that I-cells can initiate centers of aggregation and suggest compellingly that they are in fact the initiator cells for normal aggregation. The present communication extends and amplifies these findings.

A. Time lapse camera lucida drawings and photomicrographs illustrate the sequence of events during the onset of aggregation.

B. Small population samples of myxamoebae, when isolated from their neighbors shortly after deposition on washed agar, showed a distribution of aggregative centers consistent with the distribution of I-cells within the samples. Longer periods of contact with neighboring cells (including other I-cells) that surrounded the samples prior to isolation permitted progressively greater proportions of the samples to aggregate. The possibility arises of an "initiator substance" whose effect may extend over relatively great distances.

C. R-cells, incubated for long periods of time on washed agar, were found to have acquired initiative capacity. At best, only a small proportion did so and fur-



thermore could only induce the formation of aggregative centers amongst their developmental juniors (by twelve hours) but not amongst their developmental contemporaries.

## LITERATURE CITED

- ENNIS, H. L., AND M. SUSSMAN, 1958a. The initiator cell for slime mold aggregation. *Bacteriol. Proceedings*, p. 32.
- ENNIS, H. L., AND M. SUSSMAN, 1958b. The initiator cell for slime mold aggregation. *Proc. Nat. Acad. Sci.*, **44**: 401-411.
- SHAFFER, B. M., 1956. Properties of acrasin. *Science*, **123**: 1172-1173.
- SUSSMAN, M., 1956. On the relation between growth and morphogenesis in the slime mold *Dictyostelium discoideum*. *Biol. Bull.*, **110**: 91-95.
- SUSSMAN, M., 1958. A developmental analysis of slime mold aggregation. McCollum-Pratt Symposium on the chemical basis of development. (In press.) Johns Hopkins University Press, Baltimore, Md.
- SUSSMAN, M., AND E. NOEL, 1952. An analysis of the aggregation stage in the development of the slime molds Dictyosteliaceae. I. The populational distribution of the capacity to initiate center formation. *Biol. Bull.*, **103**: 259-268.
- SUSSMAN, M., F. LEE AND N. S. KERR, 1956. Fractionation of acrasin. *Science*, **123**: 1171-1172.